39-kD Protein Inhibits Tissue-type Plasminogen Activator Clearance In Vivo

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Abstract

Tissue-type plasminogen activator (t-PA) is a plasma serine protease that catalyzes the initial and rate-limiting step in the fibrinolytic cascade. t-PA is widely used as a thrombolytic agent in the treatment of acute myocardial infarction. However, its use has been impaired by its rapid hepatic clearance from the circulation following intravenous administration. Studies with both rat hepatoma MH_1C_1 cells (G. Bu, S. Williams, D. K. Strickland, and A. L. Schwartz. 1992. *Proc. Natl. Acad. Sci. USA.* 89:7427–7431) and human hepatoma HepG2 cells (G. Bu, E. A. Maksymovitch, and A. L. Schwartz. 1993. *J. Biol. Chem.* 28:13002–13009) have shown that binding of t-PA to its clearance receptor, the low density lipoprotein receptor-related protein/α_2-macroglobulin receptor, is inhibited by a 39-kD protein that copurifies with this receptor. Herein we investigated whether administration of purified recombinant 39-kD protein would alter t-PA clearance in vivo. We found that intravenous administration of purified 39-kD protein to rats prolonged the plasma half-life of ^{125}I-t-PA from 1 min to ~ 5–6 min. The plasma half-life of t-PA enzymatic activity was similarly prolonged following intravenous administration of purified 39-kD protein. In addition we found that the 39-kD protein itself was rapidly cleared from the circulation in vivo. Clearance of ^{125}I-39-kD protein was a biphasic process with half-lives of 30 s and 9 min and the liver was the primary organ of clearance. Preadministration of excess unlabeled 39-kD protein slowed ^{125}I-39-kD protein clearance in rats in a dose-dependent manner, suggesting that specific clearance receptors were responsible for this process. Administration of increasing doses of unlabeled 39-kD protein along with labeled 39-kD protein resulted in a decrease in the amount of labeled 39-kD protein associating with the liver and a concomitant increase in the amount of labeled 39-kD protein associating with the kidneys, indicating two clearance mechanisms exist for the 39-kD protein. (*J. Clin. Invest.* 1993. 92:937–944.) Key words: tissue-type plasminogen activator • 39-kD protein • low density lipoprotein receptor-related protein/α_2-macroglobulin receptor • clearance

Introduction

The low density lipoprotein receptor-related protein/α_2-macroglobulin receptor (LRP/α_2MR)[1] is a large cell surface glycoprotein that binds several unrelated ligands including α_2-macroglobulin-proteinase (α_2M*) (1, 2), apolipoprotein E-enriched β-migrating very low density lipoprotein (βVLDL) (3–6), lipoprotein lipase (7), urokinase/plasminogen activator inhibitor type-1 complex (8), *Pseudomonas* exotoxin A (9), and a 39-kD copurifying protein, also termed receptor-associated protein (RAP) (1, 9–12). LRP/α_2MR is synthesized as a single polypeptide chain of approximately 600 kD and is cleaved in a *trans*-Golgi compartment into two subunits of 515 and 85 kD, which remain associated with one another via trafficking to the cell surface (13). Metabolic pulse-chase labeling studies have demonstrated that newly synthesized 39-kD protein associates with LRP/α_2MR intracellularly. Surface labeling experiments have shown that the 39-kD protein is present on the cell surface as a complex with the 515 and 85 kD subunits of LRP/α_2MR (14). The 39-kD protein is a potent inhibitor of all known ligand interactions with the receptor, as shown by ligand blotting and by cellular uptake experiments in cultured cells expressing LRP/α_2MR (1, 8, 9, 15). In addition to copurifying with LRP/α_2MR, the 39-kD protein copurifies with glycoprotein 330 (gp330) (12), a third member of the LDL receptor gene family (16, 17) that has been identified as a receptor site for the serum protein plasminogen (18). The human 39-kD protein is the homolog of a 44-kD rat protein identified in kidney as a component of the gp330/44-kD Heymann nephritis antigenic complex (14, 19). The 39-kD protein is also the homolog of a mouse protein termed heparin-binding protein 44 (20). The physiological role of the 39-kD protein is unclear. However, the above findings have led to the proposal that in vivo the 39-kD protein may function as a regulator of LRP/α_2MR activity. The 39-kD protein may function in a similar role with regard to gp330.

We recently demonstrated from chemical cross-linking and immunoprecipitation experiments that LRP/α_2MR can function as a clearance receptor for tissue-type plasminogen activator (t-PA) in rat hepatic MH_1C_1 cells (21) and human hepatoma HepG2 cells (22) and that the 39-kD protein inhibits t-PA binding to these cells. t-PA is an endogenous plasma serine protease that converts the zymogen plasminogen to the protease plasmin. The resultant activated plasmin can proteolytically degrade the fibrin network associated with thrombi. t-PA has been used clinically to dissolve thrombi within coronary arteries associated with acute myocardial infarction (23). A major drawback to the clinical use of t-PA is its rapid plasma clearance, ranging from a $t_{1/2}$ of 1–4 min in rodents (24–29) to 5–10 min in humans (30, 31). In vivo clearance studies have shown that the liver is the primary organ responsible for the rapid uptake of t-PA from the circulation (24–31). Studies

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1. *Abbreviations used in this paper:* gp330, glycoprotein 330; GST, glutathione S-transferase; LRP/α_2MR, low density lipoprotein receptor-related protein/α_2-macroglobulin receptor; RAP, receptor-associated protein; t-PA, tissue-type plasminogen activator; α_2M*, protease- or methylamine-activated α_2-macroglobulin; βVLDL, β-migrating very low density lipoprotein.

with whole animals and with isolated liver cell preparations suggest that both oligosaccharide-dependent, mediated by mannose receptors on liver endothelial cells, and oligosaccharide-independent mechanisms, mediated via parenchymal cells, contribute to the clearance of t-PA from the circulation (32). Two distinct parenchymal cell clearance mechanisms appear to exist, based on whether or not t-PA forms a complex with plasminogen activator inhibitor type 1 (PAI-1). The relative contribution of these two systems to t-PA clearance may vary according to the physiological situation. We previously reported that free t-PA binds to LRP/α_2MR on MH_1C_1 cells (21). Orth et al. (33) demonstrated, via ligand blotting, the binding of t-PA/PAI-1 complexes to isolated LRP/α_2MR. Recently we demonstrated that t-PA/PAI-1 complexes bind to LRP/α_2MR on HepG2 cells (22). The 39-kD protein inhibits the binding of both free t-PA and t-PA/PAI-1 complexes to LRP/α_2MR. Thus, these findings suggest that LRP/α_2MR may function as a clearance receptor for t-PA in vivo and that the 39-kD protein may modulate t-PA clearance. In the present study we examined the physiology of the 39-kD protein, including its inhibitory role in t-PA clearance in vivo.

Methods

Reagents. Single-chain recombinant human t-PA expressed in Chinese hamster ovary cells was generously supplied by Genentech (lot 9124AX; South San Francisco, CA). Asialoorosomucoid and orosomucoid were from the American Red Cross. Carrier-free sodium [^{125}I]iodide was purchased from DuPont New England Nuclear (Boston, MA). IODO-GEN was purchased from Pierce Chemical Co (Rockford, IL). Glutathione-agarose, heparin-agarose, reduced glutathione and thrombin were purchased from Sigma Immunochemicals (St. Louis, MO). Hyperfilm-MP was purchased from Amersham Int'l (Buckinghamshire, UK). Sprague-Dawley rats were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Microtiter wells coated with a fibrin monomer, Glu-plasminogen, and the chromogenic substrate S-2251 were kindly provided by B. Sobel (Washington University, St. Louis, MO).

Protein iodination. Proteins were labeled with ^{125}I using the Iodogen method as described previously (34). Specific radioactivities were 5–10 μCi/μg of protein.

Purification of the 39-kD protein. A cDNA clone for the rat 39-kD protein, produced as a fusion protein with glutathione *S*-transferase (GST-39-kD protein), was kindly provided by Joachim Herz (University of Texas Southwestern Medical Center, Dallas, TX). Purification of the 39-kD protein was carried out as described previously with a few modifications (15). Briefly, DH5α bacteria harboring the GST-39-kD protein were grown at 37°C to an optical density of 0.4–0.5 at 600 nm. Expression was induced by the addition of isopropylthio-β-D-galactoside to a final concentration of 0.01%, and the cultures were grown for another 4–6 h at 30°C. Bacteria were harvested by centrifugation at 4°C and resuspended in PBS containing 1% (vol/vol) Triton X-100, 1 μM pepstatin, 2.5 μg/ml leupeptin, and 0.2 mM PMSF. Bacteria were sonicated and centrifuged at 26,000 *g* for 30 min at 4°C. The supernatant was mixed with glutathione-agarose beads at 4°C, washed in PBS, and thereafter with 50 mM Tris-HCl at pH 8. Bound GST-39-kD protein was eluted with 50 mM Tris-HCl containing 5 mM reduced glutathione at pH 8. The eluate was dialyzed against 50 mM Tris-HCl pH 8 and the fusion protein was cleaved with thrombin (Sigma Immunochemicals) in 50 mM Tris-HCl, 150 mM NaCl, and 2.5 mM $CaCl_2$ at pH 8. The free 39-kD protein was removed from the GST via heparin-agarose affinity chromatography. Following washes with 50 mM Tris-HCl, 150 mM NaCl at pH 8, the 39-kD protein was eluted from the heparin-agarose with 50 mM Tris-HCl, 400 mM NaCl at pH 8. The 39-kD protein was dialyzed against 20 mM ammonium bicarbonate pH 7.4 and lyophilized. The 39-kD protein was resuspended in sterile 0.15 M NaCl before the ligand binding assay or administration to rats.

Ligand binding assay. Rat hepatoma MH_1C_1 cells were seeded into multiwell (12 wells/plate) tissue culture plates 2 d before assay, as described previously (34). Ligand binding buffer was PBS containing 0.2 mM $CaCl_2$ and 10 mM ϵ-amino-*n*-caproic acid. Cell monolayers were washed three times on ice with prechilled binding buffer. Binding was initiated by adding 0.5 ml of binding buffer containing 4 nM ^{125}I-t-PA in the absence or presence of selected concentrations of unlabeled 39-kD protein. After incubation at 4°C for 1.5 h, buffer containing unbound ligand was removed. Cells were then washed three times with binding buffer and lysed in 0.0625 M Tris-HCl, pH 6.8, containing 0.2% (wt/vol) SDS and 10% (vol/vol) glycerol. Radioactivity of cell lysates was quantified by γ scintillation spectrometry (C5304; Packard Instrs., Meriden, CT). Total binding was determined in the presence of ^{125}I-t-PA alone. Nonspecific binding was determined in the presence of excess unlabeled t-PA (0.5 μM). Specific ligand binding was defined as the difference between total and nonspecific binding.

In vivo plasma clearance. Female Sprague-Dawley rats (weighing 200–220 g) were anesthetized with sodium pentobarbital (15 mg/200 g rat) during the time of the experiment. The indicated ^{125}I-labeled protein (30 pmol of 39-kD protein, [1.7×10^7 cpm/μg]; 30 pmol of t-PA, [1.6×10^7 cpm/μg]; 5 pmol of asialoorosomucoid [ASOR], [5×10^7 cpm/μg]; 5 pmol of orosomucoid [OR], [5×10^7 cpm/μg]) in sterile 0.15 M NaCl (total volume 500 μl) was injected into a tail vein over 30 s. In studies where unlabeled protein was administered with ^{125}I-labeled protein, the unlabeled protein was injected 1 min before injection of the ^{125}I-labeled protein. Blood samples (50–100 μl) (total vol < 800 μl) were collected from the tail artery at the indicated times into ice-cold tubes containing heparin. After collection of the 10-min sample, the chest cavity was opened and a second "10-min" sample was obtained by direct cardiac puncture. Thereafter the liver, spleen, and kidneys were removed, blotted, and weighed. ^{125}I-radioactivity was determined in a Packard gamma counter. Each of the heparinized blood samples was centrifuged, and 25 μl of the plasma fractions were spotted onto 3-mm filter paper (Whatman, Inc., Clifton, NJ), precipitated with 10% TCA, rinsed with ethanol, and ^{125}I-radioactivity was determined.

In studies of in vivo t-PA enzymatic activity, unlabeled t-PA (30 pmol) was administered intravenously either alone or following preinjection of 250 nmol of unlabeled 39-kD protein. Blood samples were collected at the indicated times as described above. (The 4-min sample was from cardiac puncture.) The resultant plasma was assayed for t-PA enzymatic activity using the solid-phase fibrin-tissue plasminogen activator activity assay (SOFIA-t-PA) of Angles-Cano (35) as modified by Sobel and colleagues (36). Briefly, plasma samples were incubated in microtiter wells coated with a fibrin monomer for 2 h at 37°C. t-PA activity was determined with 0.2 μM glu-plasminogen and 1 mM S-2251 during 2 h at 37°C. Absorbances were read at 405 nm using a Minireader II (Dynatech Labs, Chantilly, VA). Each sample was assayed in duplicate at three dilutions within the linear range of the assay.

Autoradiography. The kidneys and livers from rats injected with ^{125}I-39-kD protein in the absence or presence of 125 nmol of unlabeled 39-kD protein were fixed with 4% paraformaldehyde in PBS for 2 d. Sections of paraffin-embedded tissues were deparaffinized with xylene and rehydrated with water. Some sections were directly placed against Hyperfilm-MP for 2 wk. Other sections were covered with a photographic emulsion (NTB-2; Eastman-Kodak, Rochester, NY) for 3 d before the development with Kodak D19 developer diluted 1:1 with water. These sections were then stained with hematoxylin and eosin.

Results

39-kD protein inhibits ^{125}I-t-PA binding in rat hepatoma MH_1C_1 cells. Previously we demonstrated that the 39-kD protein generated as a fusion protein with GST-39-kD protein inhibited ^{125}I-t-PA binding to MH_1C_1 cells in a dose-dependent manner with an apparent K_i value of 5 nM (21). For these

studies, we cleaved the GST-39-kD fusion protein with thrombin and assessed the ability of cleaved and purified 39-kD protein to inhibit ^{125}I-t-PA binding to MH_1C_1 cells. Our purified preparation of recombinant 39-kD protein is seen in the inset in Fig. 1. We found that cleaved and purified 39-kD protein is a more potent inhibitor of ^{125}I-t-PA binding to MH_1C_1 cells than the GST-39-kD protein. As seen in Fig. 1, purified 39-kD protein inhibits ^{125}I-t-PA binding to MH_1C_1 cells in a dose-dependent manner with an apparent K_i of 0.5 nM.

39-kD protein inhibits ^{125}I-t-PA clearance in the rat. Fig. 2 *A* shows the plasma clearance of ^{125}I-t-PA following intravenous administration of 30 pmol of ^{125}I-t-PA in the rat. The initial plasma half-life is approximately 1 min with < 10% of the administered dose remaining in the circulation at 10 min. The plasma clearance curve seen in Fig. 2 *A* is typical of previously reported plasma clearance curves for t-PA in rats in that the rapid phase of clearance has a half-life of about 1 min. (27, 28, 32, 37, 38). Approximately 90% of the administered dose was found in the liver at 10 min. In order to determine whether t-PA clearance is a specific and saturable process, a 400-fold molar excess of unlabeled t-PA was administered to rats before the 30 pmol of ^{125}I-t-PA. Under these conditions plasma clearance of the ^{125}I-t-PA was markedly decreased with a $t_{1/2}$ of approximately 9 min, and > 40% of the administered dose remained in the circulation at 10 min. Approximately 70% of the injected ^{125}I-t-PA was associated with the liver at 10 min.

Analysis of plasma samples from in vivo clearance studies using SDS-PAGE and autoradiography demonstrated that the vast majority of the ^{125}I-t-PA (> 85%) was present as free enzyme. < 15% of the ^{125}I-t-PA was complexed with PAI-1 at all time points examined (0–10 min) (data not shown). These results are in agreement with those of Fuchs et al., who also demonstrated that t-PA is cleared from the circulation as free t-PA and not in a complex with PAI-1 (29).

Clearance experiments were performed in order to determine whether preadministration of purified 39-kD protein to rats would alter ^{125}I-t-PA clearance. As seen in Fig. 2 *A*, intravenous administration of 250 nmol of purified 39-kD protein prolonged the plasma half-life of ^{125}I-t-PA from 1 min to approximately 5–6 min. Over 30% of the administered ^{125}I-t-PA remained in the blood at 10 min. ~ 80% of the injected ^{125}I-t-PA was associated with the liver at 10 min.

Additional plasma clearance studies with well-characterized ligands (e.g., orosomucoid and asialoorosomucoid) were performed as controls. As seen in Fig. 2 *B*, the parent compound, orosomucoid, is very slowly cleared from the circulation in the rat with a $t_{1/2}$ of ~ 90 min. However, upon removal of the terminal sialic acid residues, the resultant asialoorosomucoid is cleared very rapidly with a $t_{1/2}$ of ~ 30 s. These results are essentially identical to those seen earlier with these ligands (39).

In order to determine whether administration of the 39-kD protein would alter the enzymatic activity of t-PA in vivo, unlabeled t-PA was administered to rats intravenously either alone or following preadministration of purified 39-kD protein. Thereafter plasma samples were assayed for t-PA enzymatic activity as described in Methods. Preliminary studies in vitro demonstrated, using the same molar ratio of t-PA to 39-kD protein used in vivo, that the 39-kD protein did not alter t-PA enzymatic activity (data not shown). Fig. 3 demonstrates

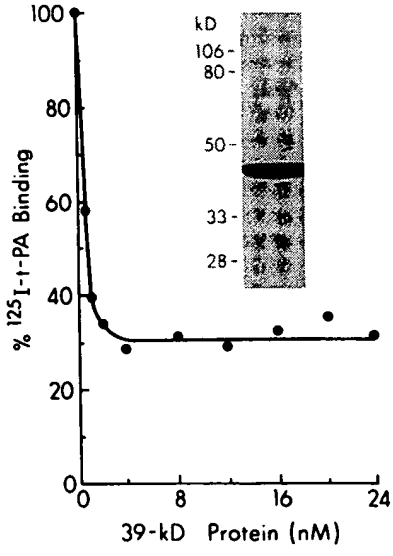

Figure 1. Inhibition of ^{125}I-t-PA binding to rat hepatoma MH_1C_1 cells by 39-kD protein. Binding of ^{125}I-t-PA (4 nM) to MH_1C_1 cells was performed in the absence or presence of various concentrations of 39-kD protein for 1.5 h at 4°C as described in Methods. 100% binding was determined in the absence of 39-kD protein. Each symbol is the average of triplicate determinations. Nonspecific binding, determined in the presence of 0.5 μM unlabeled t-PA, has been subtracted from each point. Inset, 10% SDS-PAGE of 10 μg of purified 39-kD protein stained with Coomassie brilliant blue. Molecular weight markers in kD are indicated on the left.

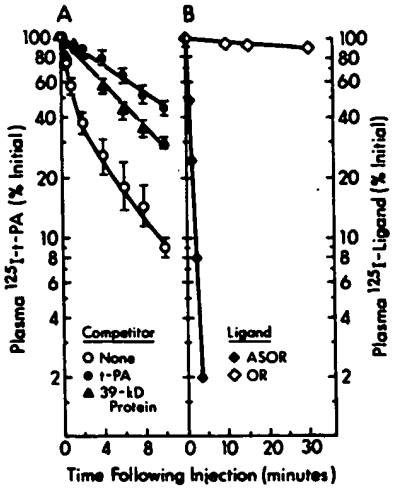

Figure 2. A. Effect of 39-kD protein or unlabeled t-PA on the plasma clearance of ^{125}I-t-PA in rats. As described in Methods, rats were injected with 30 pmol of ^{125}I-t-PA without (○) (n = 8 rats) or with preinjection of 250 nmol of unlabeled 39-kD protein (▲) (n = 4 rats) or 12 nmol of unlabeled t-PA (●) (n = 3 rats). Blood samples were collected at the indicated times and trichloroacetic acid-insoluble radioactivity was determined. Each symbol represents the mean±SEM. *B.* Plasma clearance of control proteins. Rats were injected with 5 pmol of ^{125}I-orosomucoid (OR) (◇) or 5 pmol of ^{125}I-asialoorosomucoid (ASOR) (◆). Samples were processed as described above.

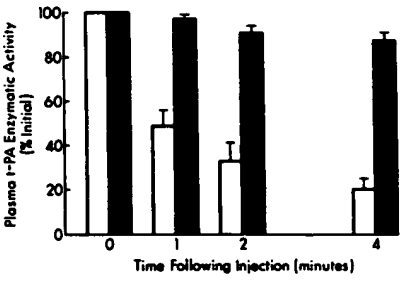

Figure 3. Effect of 39-kD protein on the enzymatic activity of t-PA in rats. As described in Methods, rats were injected with 30 pmol of unlabeled t-PA (*open bars*) (n = 4 rats) or with preinjection of 250 nmol of unlabeled 39-kD protein (*closed bars*) (n = 3 rats). Blood samples were collected at the indicated times, and plasma t-PA enzymatic activity was assayed as discussed in Methods. Each bar represents the mean±SEM. □, −39 kD protein; ■, +39 kD protein.

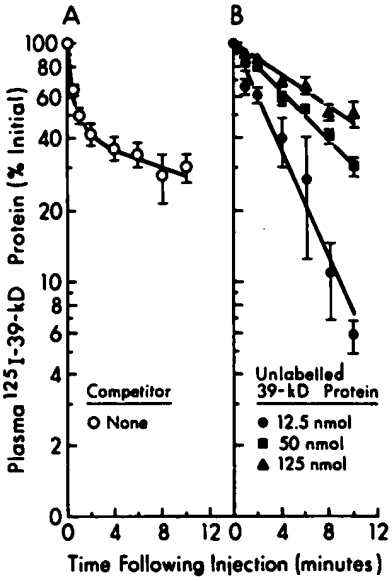

Figure 4. Effect of unlabeled 39-kD protein on the plasma clearance of ^{125}I-39-kD protein in rats. In *A*, rats were injected with 30 pmol of ^{125}I-39-kD protein (○) (n = 6 rats). In *B*, 12.5 nmol of unlabeled 39-kD protein (●) (n = 3 rats), 50 nmol of unlabeled 39-kD protein (■) (n = 4 rats), or 125 nmol of ^{125}I-39-kD protein (▲) (n = 2 rats) was injected 1 min before injection of the radiolabeled 39-kD protein. Blood samples were drawn at the indicated times, and radioactivity was determined as described previously. Each symbol represents the mean±SEM.

that plasma t-PA enzymatic activity decreases with the identical kinetics seen in Fig. 2 *A* for ^{125}I-t-PA (i.e., initial plasma half-life of ~ 1 min). Fig. 3 also shows that intravenous administration of the 39-kD protein prolongs the plasma half-life of t-PA enzymatic activity from 1 min to ~ 9 min.

39-kD protein is rapidly removed from the circulation. The 39-kD protein specifically binds to and is rapidly endocytosed by rat hepatoma MH_1C_1 cells (S. P. Iodonato, G. Bu, E. A. Maksymovitch, and A. L. Schwartz, submitted for publication). We (unpublished observations) and others (14) have been unable to identify extracellular unbound 39-kD protein. These observations prompted us to examine the clearance of the 39-kD protein in vivo. Fig. 4 *A* shows that the 39-kD protein is rapidly cleared from the circulation in the rat. The clearance of 30 pmol of ^{125}I-39-kD protein occurs as a biphasic process with half-lives of ~ 30 s and 9 min. 60% of the injected ^{125}I-39-kD protein is cleared within 2 min. 28% of the administered dose remained in the circulation at 10 min. ~ 70% of ^{125}I-39-kD protein was associated with the liver and 7% of ^{125}I-39-kD protein was associated with the kidney at 10 min (Table I). To determine whether the ^{125}I-39-kD protein clearance was specific and saturable, increasing doses of unlabeled 39-kD protein were administered to rats. Fig. 4 *B* demonstrates that administration of unlabeled 39-kD protein slows ^{125}I-39-kD protein clearance in a dose-dependent manner. Doses of 12.5, 50, and 125 nmol of unlabeled 39-kD protein resulted in plasma half-lives of ^{125}I-39-kD protein of 3, 6, and 9 min, respectively. At 10 min, 72%, 25%, and 23% of ^{125}I-39-kD protein was associated with the liver, whereas 12%, 13%, and 31% of ^{125}I-39-kD protein was associated with the kidney following administration of 12.5, 50, and 125 nmol of unlabeled 39-kD protein, respectively (Table I). The remaining fraction of ^{125}I-39-kD protein not found in the liver and kidney at 10 min was still in the circulation (Fig. 4). There was no change in the fraction of ^{125}I-39-kD protein found in the spleen (1.7–2.5%) under any of these conditions (Table I).

Clearance capacity of the liver and kidney for the 39-kD protein. Receptor-mediated protein clearance systems are well described for several circulating proteins (39–42). Receptor-mediated endocytosis describes a process whereby extracellular proteins are trafficked into the cell and ultimately are released into the extracellular milieu either intact or degraded. This process requires more than 20 min in liver, kidney, and other tissues (43, 44). Therefore a measure of an organ's capacity to clear a given protein from the circulation can be assessed independently from degradation and/or cellular release by determining the organ distribution of the protein at 10 min. Thus, we assessed the organ distribution of the 39-kD protein 10 min after its intravenous administration. As seen in Table I, the liver is the major organ of 39-kD protein clearance following administration of 30 pmol of ^{125}I-39-kD protein. Administration of increasing amounts of unlabeled 39-kD protein along with the 30 pmol of ^{125}I-39-kD protein resulted in a decrease in the amount of ^{125}I-39-kD protein cleared by the liver and a concomitant increase in the amount of ^{125}I-39-kD protein cleared by the kidney (Table I). Table I also shows the clearance capacities of the liver and kidney for the 39-kD protein. At the maximum dose of 39-kD protein examined (i.e., 125 nmol), the liver, 10 min after intravenous administration, had cleared 29 nmol of 39-kD protein, whereas the kidneys had cleared 39 nmol (Table I). The remaining 39-kD protein was in the blood (i.e., as seen in Fig. 4 *B*, ~ 50% or 60 nmol of the administered dose remained in the blood at 10 min).

Sites of 39-kD protein clearance in the kidney and liver. Tissue sections of kidney and liver (Figs. 5 *A* and *C*, respec-

Table I. Tissue Distribution of ^{125}I-radioactivity following Intravenous Injections of ^{125}I-39-kD Protein in the Absence or Presence of Unlabeled 39-kD Protein

n	^{125}I-39-kD protein	Unlabeled 39-kD protein	% ^{125}I-radioactivity/organ			10-min clearance (nmol/organ)	
			Liver	Kidney	Spleen	Liver	Kidney
6	30 pmol	—	69±5	7.3±1	1.7±0.2	0.021	0.002
3	30 pmol	12.5 nmol	72±3	12±1	2.5±0.2	9.0	1.5
4	30 pmol	50 nmol	25±6	13±2	2.0±0.6	12.5	6.5
2	30 pmol	125 nmol	23±5	31±10	2.2±0.3	29	39

Rats were injected with 30 pmol of ^{125}I-39-kD protein as described in Methods. Varying amounts of unlabeled 39-kD protein were injected 1 min before injecting radiolabeled 39-kD protein. After 10 min, the animals were killed, organs were obtained, and their content of ^{125}I-radioactivity was determined by gamma counting. n, number of animals in each group. Data are expressed as mean±SEM.

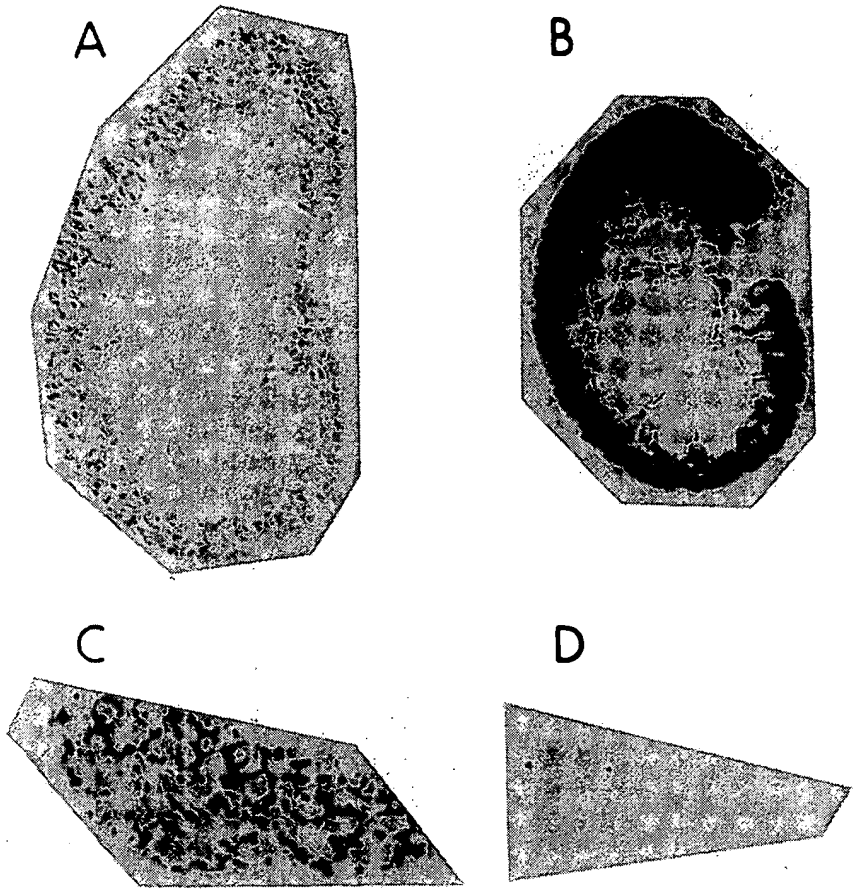

Figure 5. Autoradiograms of liver and kidney sections from rats injected with ^{125}I-39-kD protein in the absence or presence of unlabeled 39-kD protein. In *A* and *C*, rats were killed 10 min after administration of 30 pmol of ^{125}I-39-kD protein. The kidneys and liver were dissected out and fixed with 4% paraformaldehyde in PBS for 2 d at 4°C. Paraffin-embedded sections of kidney (*A*) and liver (*C*) were prepared and exposed directly to film. In *B* and *D*, 125 nmol of unlabeled 39-kD protein was injected into rats 1 min before injecting 30 pmol of ^{125}I-39-kD protein. 10 min after the injection, the kidneys (*B*) and liver (*D*) were removed and processed as described above.

tively) showed that in vivo administration of 30 pmol of ^{125}I-39-kD protein resulted in the appearance of radioactivity in the renal cortex and in a trabeculated pattern in the liver. When 125 nmol of unlabeled 39-kD protein was administered 1 min before the 30 pmol of ^{125}I-39-kD protein, the appearance of radioactivity in the renal cortex increased approximately fourfold (Fig. 5 *B*), consistent with the results seen in Table I. The pattern of radioactivity in the liver (Fig. 5 *D*) appeared more homogeneous and of substantially less intensity when compared with the liver of the rat following ^{125}I-39-kD protein only.

Kidney clearance may be via glomerular filtration and/or cellular uptake. Therefore we examined the kidney by microscopic autoradiography 10 min following the 39-kD protein administration. The hematoxylin- and eosin-stained autoradiograms of kidney sections from rats administered with ^{125}I-39-kD protein in the absence or presence of 125 nmol of unlabeled 39-kD protein appeared similar. Figs. 6, *A* and *B* show kidney sections from a rat administered with 30 pmol of ^{125}I-39-kD protein. The majority of grains appeared within Bowman's space (Fig. 6 *A*) and within the lumen of the proximal tubules (Fig. 6 *B*). Only a minor fraction of the grains were cellular. Cortical glomeruli contained more grains than juxtamedullary glomeruli (data not shown).

Liver sections were also examined by autoradiography (Fig. 6 *C* and *D*). The grains in the liver sections of rats that received only ^{125}I-39-kD protein (Fig. 6 *C*) were predominantly cellular. However, in rats that received 125 nmol of unlabeled 39-kD protein before the ^{125}I-39-kD protein (Fig. 6 *D*) the vast majority of grains were within the sinusoidal lumen.

Discussion

t-PA is a serine protease that catalyzes the initial and rate limiting step in the fibrinolytic cascade by converting the zymogen plasminogen to plasmin. t-PA is used widely as a fibrinolytic agent in the treatment of acute myocardial infarction (23). One substantial limitation to the clinical use of t-PA is its rapid plasma clearance (half-life is ~ 1–5 min), which is predominantly due to an active uptake system in the liver (24–31). Recent studies using both human and rat hepatoma cells have demonstrated that LRP/α_2MR is an hepatic clearance receptor for both free t-PA (21) and t-PA/PAI-1 complexes (22). The 39-kD protein is a potent inhibitor of all known ligand interactions with LRP/α_2MR, including t-PA and t-PA/PAI-1 complexes, as shown by both ligand binding and by ligand uptake experiments in cultured cells (1, 2, 6, 15, 21, 22). Because the 39-kD protein inhibits ^{125}I-t-PA binding to the t-PA receptor in rat hepatoma MH_1C_1 cells, we administered this protein to rats just before injecting ^{125}I-t-PA and found that the 39-kD protein prolonged the half-life of t-PA five- to sixfold. We also found that the plasma half-life of t-PA enzymatic activity was similarly prolonged following intravenous administration of the 39-kD protein. These results suggest that the 39-kD protein decreases ^{125}I-t-PA clearance in rats by inhibiting t-PA from binding to LRP/α_2MR.

The deduced amino acid sequence of the 39-kD protein contains a putative signal sequence suggesting that the 39-kD protein may be secreted. However we and others have been unable to detect secreted 39-kD protein (14). We recently

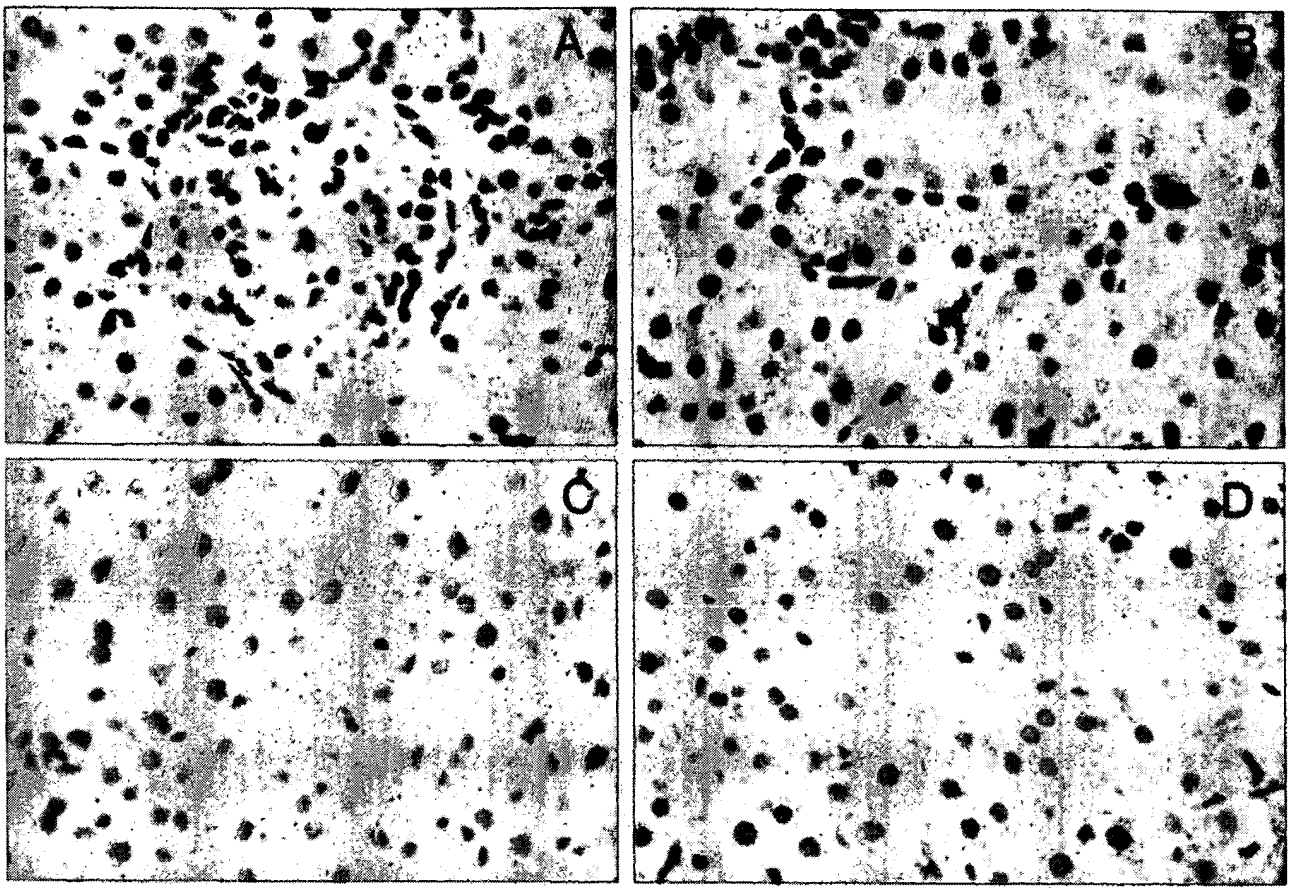

Figure 6. Autoradiograms of hematoxylin and eosin stained liver and kidney sections from rats injected with the 39-kD protein. In *A* and *B*, 125 nmol of unlabeled 39-kD protein was injected into rats 1 min before injecting 30 pmol of ^{125}I-39-kD protein. After 10 min, the kidneys were removed, and paraffin-embedded sections were processed for autoradiography and stained with hematoxylin and eosin as described in Methods. Liver sections from rats injected with 30 pmol of ^{125}I-39-kD protein in the presence (*C*) or absence (*D*) of 125 nmol of 39-kD protein were processed as described above.

found that the 39-kD protein specifically binds to and is endocytosed by hepatic MH_1C_1 cells (S. P. Iodonato, G. Bu, E. A. Maksymovitch, and A. L. Schwartz, submitted for publication). These results therefore suggested that the 39-kD protein may be cleared in vivo. Indeed, as seen in Fig. 4, 39-kD protein clearance is rapid and specific. The nature of the slower phase of 39-kD protein clearance is not clear. It may, however, represent a small (i.e., < 10 pmol) pool of 39-kD protein binding or sequestration sites (a serum protein or cellular blood component, for example, LRP/α_2MR on monocytes [45]). Preadministration of > 10 pmol of unlabeled 39-kD protein would mask these sites, and ^{125}I-39-kD protein clearance would linearize. We were unable to detect serum component(s) that bound the 39-kD protein. However we did determine that ~ 15% of the ^{125}I-39-kD protein (5 pmol) associated with blood cells (data not shown). These results suggest an additional unknown mechanism must also exist.

Similar to earlier studies (23–30), the tissue distribution of ^{125}I-t-PA radioactivity seen in the present study indicates that the majority of t-PA is cleared by the liver. Although LRP/α_2MR is expressed in many different tissues (46), its endocytic function appears to be expressed predominantly in the liver. When injected into the circulation, specific ligands for LRP/α_2MR are predominantly cleared by the liver (47), which is also the major site of t-PA clearance. We propose that LRP/α_2MR functions in vivo as a clearance receptor for t-PA. We recently found that antibodies generated against LRP/α_2MR could inhibit t-PA binding to hepatic cells (22). Whether antibodies to LRP/α_2MR alter t-PA clearance in vivo remains to be established.

The tissue distribution of ^{125}I-39-kD radioactivity suggests there are two sites of clearance for the 39-kD protein, one residing in the liver and another in the kidney. Since the 39-kD protein binds to LRP/α_2MR with high affinity (2), we propose that LRP/α_2MR functions as a high affinity clearance receptor for the 39-kD protein in liver. The autoradiograms of ^{125}I-39-kD protein clearance in liver indicate that hepatic uptake of the ^{125}I-39-kD protein occurs largely on hepatocytes. Renal clearance may be glomerular filtration and/or cellular uptake. Our autoradiograms of kidneys from rats injected with the ^{125}I-39-kD protein show the majority of ^{125}I-39-kD protein in Bowman's space and the lumen of the proximal tubules. Only a minor fraction of the ^{125}I-39-kD protein was cellular. It is not clear at present whether the 39-kD protein passes from the circulation into Bowman's space and the proximal tubules simply by filtration or whether a component of protein-mediated transport is involved. It is also not clear at present to what extent the 39-kD protein is reabsorbed back into the circula-

tion or excreted in the urine. If renal clearance of the 39-kD protein is protein mediated, one likely candidate is glycoprotein 330 (gp330), a large molecular weight glycoprotein that shares considerable sequence identity with LRP/α_2MR (16) and that binds the 39-kD protein with high affinity (12). gp330 has been localized, via immunoelectron microscopy, to clathrin-coated pits both in the proximal tubule brush border and in the glomerular epithelium (19, 48, 49). The localization of gp330 to clathrin-coated pits of the tubule epithelial cells suggests that gp330, like LRP/α_2MR, may be involved in the endocytic internalization of ligands. Since the sites of 39-kD protein clearance and gp330 localization coincide, gp330 may well play a role in 39-kD protein clearance in the kidney. Further studies of the physiology of 39-kD protein interaction in the kidney in vivo will be necessary to further resolve this issue.

Our findings thus demonstrate that recombinant 39-kD protein, when injected into rats, can markedly decrease t-PA clearance and prolong t-PA enzymatic activity via inhibition of the hepatic t-PA receptor, LRP/α_2MR. In addition, we have demonstrated that this regulatory ligand, the 39-kD protein, is rapidly cleared from the circulation and is taken up by both the liver and kidney.

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